Aseptic Transfer and Serial Dilution

Based on an exercise created by Bob Griffin, rewritten by Dr. Raymond R. White and Crima Pogge

A. Objectives

On completion of this exercise you should be able to do all of the following:

1. Distinguish between sterility and asepsis.
2. Demonstrate and/or describe aseptic techniques for pipetting with a serological pipette and a micropipettor, serial dilution, and spreading of an inoculum on a petri plate.
3. List the principal sources of microbial contamination encountered in the laboratory and briefly describe the principal apparatus and procedures used to minimize the probability of contamination, including at least the Bunsen burner, petri dish, Morton closure, culture tube, pipette, and liquid disinfectant.
4. Explain the procedures followed for safe disposal of contaminated materials.
5. Given an initial concentration and a set of serial dilutions made from it, calculate the final concentration.
6. Given a set of serial dilutions and the final concentration produced by those dilutions, calculate the initial concentration.
7. State clearly the scientific question that each part of this exercise is designed to test.
8. Given the weight of some solid material from which a solution, extract, or suspension is made and the volume of solvent used, and given a set of serial dilutions thereof and the final concentration of some substance or organism in the final dilution, calculate the amount of that substance in the original sample of solid.

B. Before coming to lab

1. Review the materials on the serological pipette and the micropipettes.
2. Obtain a passing score on the quiz on the use of those instruments.
3. Write the cryptic title “Aseptic Transfer and Serial Dilution’ at the top of a fresh page in your laboratory notebook.
4. Read the Experimental Method section which follows. Construct a protocol, including a list of exactly how many tubes and plates of which media you will need.
5. Write that protocol in your laboratory notebook just below the cryptic title.
6. Write the cryptic title in the table of contents of your notebook and leave three blank lines after it for later insertion of a descriptive title.

C. During lab, first lab period
Show your completed protocol to your instructor and state what materials you need and how many of each.

1. A. Take a sterile cotton swab, wipe it across a reasonable area of the surface of your laboratory table to pick up any microorganisms which might be there and streak the agar surface of ONE HALF of a sterile nutrient agar plate with that swab. I suggest treating a side bench, as earlier labs have done the tables. Be sure to prevent contamination from any other source. Be sure to label that half of the plate. Each person does this.
1. B. Disinfect your area of the table with one of the disinfectans available. Spread the disinfectant with a piece of clean paper towel. Be sure the table surface is thoroughly wet and is allowed to air-dry. Do not wipe it dry. Do not leave any puddles or you will be waiting all day for it to dry. Wet it thoroughly, then wipe it to the point where there is a very thin film of disinfectant. When it is absolutely dry, streak the area you disinfected with a fresh sterile swab and then use that swab to streak the other half of the plate you used in part 1.A above. Be sure to label your plate so you can positively identify it later. Record in your notebook the chemical name of the disinfectant. Each person does this.
2. Take a second sterile nutrient agar plate, lightly touch several unwashed fingers to the agar surface (the pads of the fingers, not the nails), and replace the cover. Label that half. Then wash your hands thoroughly with soap and touch the same fingers (shake off excess water, air dry to dampness) to the agar surface of the other half of the same plate. Record in your notebook a complete identification of the soap used. Each student will do a plate for this one.
3. Sign up on the chart by the chalkboard for any ONE of the following, then carry it out.

You are assigned to the group (A-E) with the fewest students already signed up.

A. Label a plate and then leave it uncovered for 5 minutes. Cover, & incubate.
B. Label a plate and then leave it uncovered for 10 minutes. Cover, & incubate.
C. Label a plate and then leave it uncovered for 20 minutes. Cover, & incubate.
D. Label a plate and then leave it uncovered for 40 minutes. Cover, & incubate.
E. Label a plate and then leave it uncovered for 80 minutes. Cover, & incubate.

4. Perform a 5-fold serial dilution of the *E. coli* broth supplied to you (see note 3 and Figure 1 below). Work in pairs observing each other, EACH doing one five-fold dilution.
   - Take one ml of broth from the original *E. coli* culture and add it to a 9ml sterile water blank to achieve the first dilution to 0.1 relative to the original concentration of bacteria.
   - Vortex or otherwise mix the bacteria in the new tube to approximate an even concentration.
   - Continue (repeat) to obtain a 10^{-4} concentration; for the last dilution use a tube of 9ml sterile broth instead of water.

- Spread 0.1ml from the broth tube on one plate and 0.1ml from the previous water blank onto another plate.
- Label: name, dilution, lab section.

5. Prepare a streak plate according to note 4 and Figure 2 below.
6. Incubate the two plates and the final broth tube for analysis in the next lab period.
7. Double-check all your plates and the tube of broth to be sure they are labeled. The plates are to be bound up in a stack which will have your name on it. Your tube is to be incubated in a rack along with all the tubes for your entire laboratory section so you should have your initials or some other identifying mark on your tube so you can recognize it even if someone moves the rack or rearranges the tubes.

Do not incubate the water blanks. They do not contain any nutrient; therefore there will be no significant growth in them. However, the value of a simulation is in making the correct procedures a matter of habit, so continue the simulation and dispose of the water blanks AS IF THEY WERE CONTAMINATED WITH DANGEROUS MICROBES!

1. Leave all the closures on the tubes.
2. Remember that the closures will leak, so you must NOT tilt or shake the tubes.
3. Do not dump anything out. We don't want the sink contaminated.
4. Do not return the water blanks to the basket they came from to mislead other students into thinking they are sterile. Some people do not find this point as obvious as you do.
5. Remove all labels from the water blanks.
6. Place them —- standing up, so they don't leak —— in one of the baskets in the biohazard disposal area. Pack one basket thoroughly with tubes before starting on another one. As a basket fills, straighten the tubes to the vertical position. Do not start another basket until the first one is fully packed with tubes all standing vertically and parallel.
7. All biohazards are sterilized in an autoclave (a steam pressure sterilizer) before they are opened for cleaning or discarded into the trash stream.

D. During lab, second lab period

1. Remove your plates and tubes of broth from the incubator and examine them carefully. Record whether each tube of broth is clear or cloudy. For each plate, record the number of different types of colonies and the number of each type.
2. After you have recorded your data in your laboratory notebook, determine the total number of colonies for each plate and enter those totals on the class data chart.
3. Remove all labels from your tubes and place them the proper biohazard disposal container. DO NOT CONTAMINATE THE SINK BY POURING OUT THE BROTH. DO NOT CONTAMINATE THE DISPOSAL TRAY BY

ALLOWING YOUR TUBES TO LIE DOWN. STAND THEM UP IN A BASKET AND BE SURE TO PACK ONE BASKET COMPLETELY BEFORE STARTING ON ANOTHER.
You do not need to remove your labels from your petri plates unless they are glass. The plastic ones we usually use melt when they are autoclaved, so cannot be re-used. You simply place them in the large, red Biohazard bag provided for safe disposal.

E. After the second lab period

Once you have received a copy of the class data:

1. Prepare the statement of Conclusions described on page xxx of these instructions and write it in your notebook.
2. Fasten the copy of the class data sheet correctly into your laboratory notebook .
3. Compose a descriptive title for the investigation. Enter it into your table of contents.

F. Background

Note 1: Avoiding contamination with pathogens

Sepsis is defined as the state or condition of poisoning with pathogenic (disease-causing) microorganisms, especially bacteria or fungi. Asepsis is the opposite of sepsis; that is, asepsis is the absence of sufficient pathogenic organisms to cause any significant problem. By extension, the term is commonly used to include any handling of any material which attempts to minimize contamination by microorganisms from the environment, or contamination of the environment with microorganisms which might be contained in a tube or flask that is being handled. Aseptic techniques are often--though incorrectly— referred to as “sterile technique”; absolute sterility —— the total absence of any living organism — is extremely difficult to maintain, especially in any container that must be opened for the addition or removal of some material. We try for sterility, but asepsis is the best we can routinely expect.

In order to avoid contamination, you must be aware of its sources. All of your glassware and its contents will have been sterilized ahead of time for you, but three other sources of contamination with which you do have to concern yourself are:

1. the lab benches where you work,
2. your fingers and other parts of your body and clothing, and
3. the air.

Disinfecting the table surface is, of course, a routine part of aseptic technique, and its importance will be brought out by the two plates you will swab at the very beginning of this exercise. Contamination from your fingers, etc. is easily prevented by being careful what you touch. The air, however, is more difficult to deal with.

Disinfecting your lab bench at the beginning of a laboratory period helps reduce air-borne contamination because it kills microbes on the table before you stir them up into the air, but the air will still contain a few. If all the doors and windows are closed, the movement of the air is greatly reduced and most of the microorganisms settle out. If they are settling out in still air, they settle out straight down for the most part, and keeping your sterile material covered all the time will prevent contamination. When something must be opened, you can greatly reduce the possibility of contamination by keeping the container covered from above and the opening through which you add or remove something as much to the side as possible, rather than open in an upward direction. Tubes are inclined, petri dishes have their covers just barely lifted. Tubes and flasks have their mouths passed through a Bunsen burner flame for a second or so before their closures are replaced.

Pipettes are supplied already sterilized inside a closed canister. They will remain sterile until you open the canister. They will become thoroughly contaminated if you take them out prematurely and walk around the room with them or let them touch your clothing or some part of your body or the table.

The pipettes must be slid within the canister, with the cover still on, until the pipettes are as far toward the cover as they can go; the pipette canister must thereafter be kept on its side, the cover must be off for as short a time as possible, and you should touch only the mouthpiece end of the pipette (with throughly clean hands, of course). When you are finished with a pipette, lower it into one of the tall, white, plastic cylinders of disinfectant. The instructor will demonstrate.

Note 2: Standard methods for growing/observing microorganisms

Broth cultures (in tubes)

Microorganisms are inoculated into a sterilized broth that provides the appropriate physical and chemical environment for the growth of the microorganism in question (if you do not know the requirements, you need to experiment with mixing different nutrients and growth factors). When you grow microorganisms in an appropriate broth, they often grow in a way that is characteristic for that microorganism. For example, some microorganism increase the turbidity (or cloudiness) of the broth, others will only grow at the surface and form a mat of cells called a **pellicle**.

We use **Morton closures** to protect the cultures from aerosol contamination while allowing for gas exchange between the interior of the tube and the air outside. This means: the closures do not seal the tubes. **You must not tip of shake the tubes**.

Agar plates (Petri dishes)

Microorganisms that need more oxygen are inoculated onto an agar-agar medium in a Petri dish. Agar-agar is derived from seaweed and nutrients and growth factors can be mixed into it. Petri dishes have covers which fit over the top and down the sides to prevent aerosol contamination by allowing gas exchange. Agar plate are also used for easier observation of microbial colonies. If pure colonies grow on agar surfaces, they often exhibit shapes, textures, and colors characteristic for those microorganisms.

Once you have inoculated your agar plates you need to wait a few minutes to let the microbes attach to the agar, then you need to i**nvert** the plates to prevent condensation from affecting your results.

Note 3: Serial dilution

Serial dilutions can be used to calculate the concentration of microorganisms. As it would usually be impossible to actually count the number of microorganisms in a sample, the sample is diluted and plated to get a reasonable number of colonies to count. Since each colony on an agar plate theoretically grew from a single microorganism the number of colonies (or **CFU**s - Colony Forming Units) is representative of the number of viable microorganisms. Since the dilution factor is known, the number of microorganisms per mL in the original sample can be calculated.

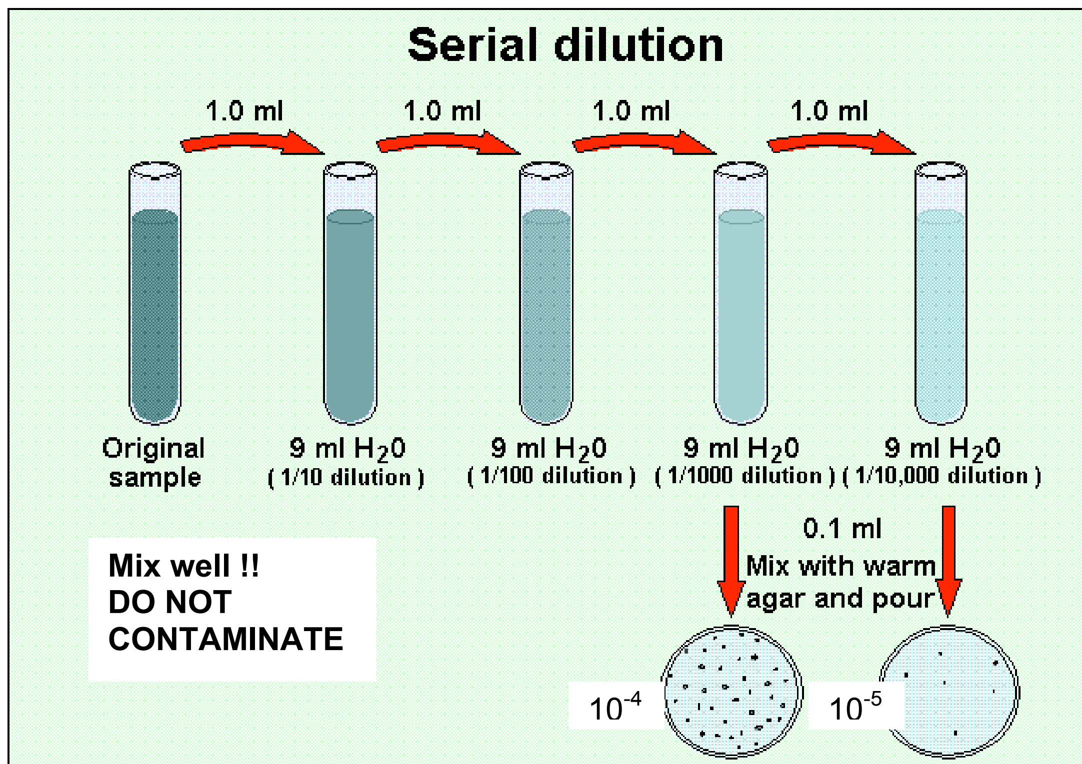


Figure 1: Example of serial dilution (**Note:** this example is of a four-fold dilution)

Remember: no individual colonies visible, all grown together = lawn
> 300 colonies => too many to count
< 30 colonies => error too big when extrapolating CFUs/ml both
159 colonies on the 10^5 plate = 1.59×10^7 CFU/ml in original sample
CFU = colony forming unit

Note 4: Streaking for isolation
(from: R. Hurlbert, 1999 at
http://www.slic2.wsu.edu:82/hurlbert/micro101/pages/101lab3.html)

Bacteria rarely exist alone in nature. A collection of a single type of bacteria isolated and growing free from all other microbes is called a pure culture (PC). Microbiologists almost always study PC of bacteria because it is the only way to learn about the morphology and physiology of individual bacterial species. The most common technique to obtain a PC involves rubbing a mixture of bacteria across a solid surface of some material that the bacteria will grow on. Usually this is a sterile plate of bacterial medium (food) that has been solidified with a material made from seaweed. This solidifying material is called agar agar (this is not a misprint, however we will refer to it simply as agar from now on).
The streaking technique consists of picking up a bacterial mixture on the end of a sterile wire loop and rubbing it rapidly across approximately 1/4 of the surface of a sterile agar-plate. The loop is then sterilized, cooled and streaked at approximately a right angle beginning in the end of the *first streak*. Again approximately 1/4 of new surface is covered by the *second streak*. The process is repeated twice more, each time rubbing the freshly sterilized loop into the end of the previous streak to pick up a few cells and streaking these cells into a new, virgin area of the plate. In this way the various microbes in the original mixture are *randomly rubbed off* of the loop and deposited as single cells onto the solid surface of the medium (Fig. 2).

The individual cells then grow and produce bacterial colonies (a visible mass of bacteria) which supposedly are pure cultures (PCs). Actually, a microbiologist rarely trusts the first streaking to give them a PC, so they pick an isolated colony from the first streak plate and repeat the streaking process once or twice more to be absolutely certain that they really have a PC. Your instructor will demonstrate this technique. You will then streak a mixed culture on an agar-medium plate. Following colony growth you will examine some of the *possible* pure culture colonies.

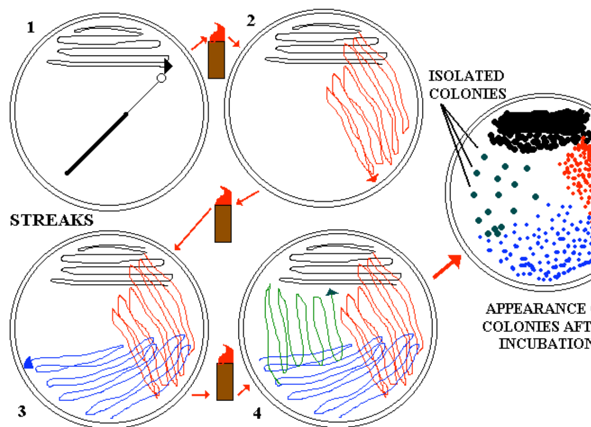


Figure 2. Illustration of streaking for purification.

STREAKING FOR ISOLATION

1. Label the bottom of the plate with your name, the sample name and date.
2. Practice streaking on the open palm of your hand or with sharpie on a circle on paper the size of a petri dish.
3. Sterilize/cool your loop.
4. Dip the sterile loop into the culture and withdraw a loop of the suspension.
5. Gently lift the lid of your petri dish and hold it over your agar plate.
6. Hold the loop parallel to the agar surface and lay it gently on the agar, tipped slightly up, about 0.5 cm from the edge of the plate and, without lifting the loop, wipe it lightly back 'n forth across the agar surface while drawing the loop slowly across 1⁄4 of the plate; each sweep of the loop should be in a new or virgin area of the agar. Continue until you have streaked about 1⁄4 of the agar surface.
7. Sterilize/cool the loop.
8. Rub the sterile loop across the END of the previous series of streaks two or three times to pick up a few microbes. Then begin streaking as before into a *fresh area* of the plate. Stop when you have covered another 1/4 of the plate's surface (Fig. 1 #2).
9. Repeat steps # 5 & #6 *two more times*, at which point there should be 4 streaks on the agar surface as shown in the illustration above and 85 to 95% **OF THE PLATE SURFACE SHOULD BE STREAKED**.
10. Place your plate UPSIDE down in the box provided.

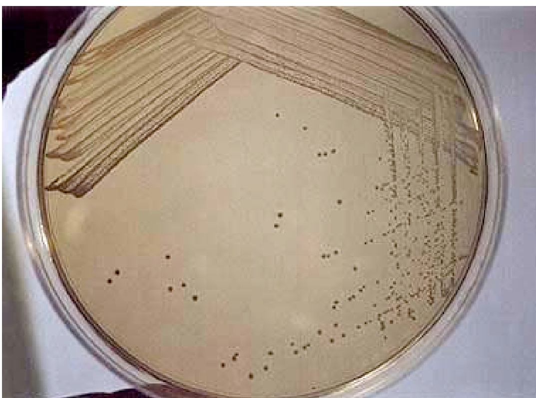
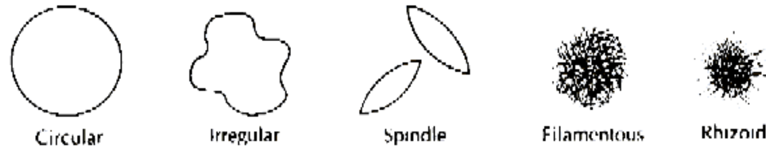


Fig. 3: Example of excellent streak plate result.

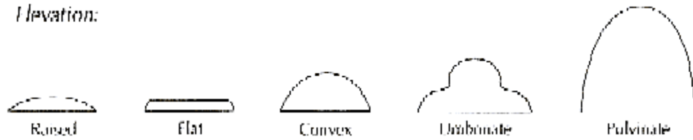
Note the isolation of colonies and the absence of contamination.

Note 5: Diversity and growth patterns of microorganism and colonies

I. Colony morphology
   A. Colony surface: smooth; rough; dull; glistening; wrinkled; contoured; granular
   B. Optical characteristics: opaque; translucent; dull; glossy; iridescent; opalescent
   C. Consistency: butyrous; membranous; viscid; brittle
   D. Pigmentation: any color; soluble or non-soluble
   E. Forms: circular; irregular; spindle; filamentous; rhizoid



   F. Elevation: raised; flat; convex; umbonate; pulvinate



   G. Margins: entire; undulate; lobate; filamentous; curled; spreading

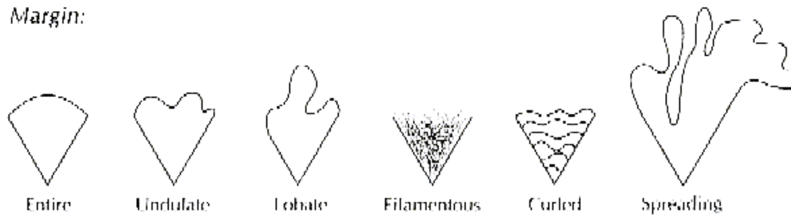


Figure 4 shows bacterial growth patterns in broth culture tubes.

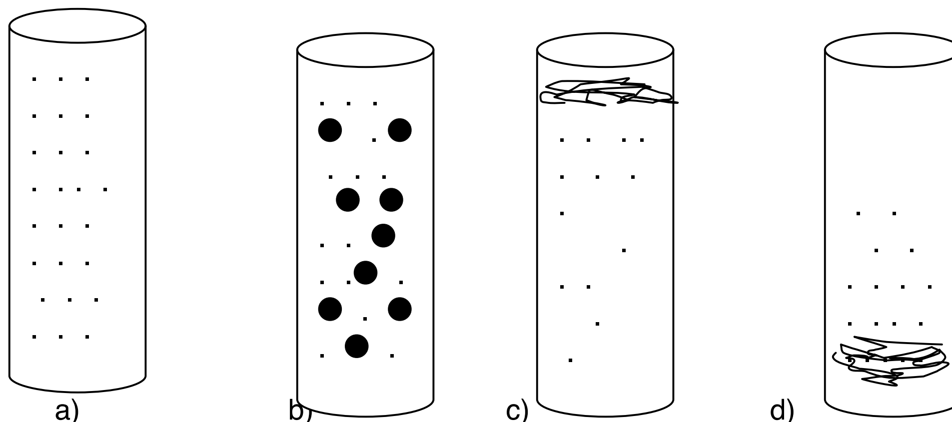


Figure 4: Bacterial growth in broth culture tubes
a) uniform, fine turbidity;
b) flocculant growth;
c) pellicle; d) sediment

G. Review questions

1. What question do we try to answer with the serial dilution?

2. What are maximum and minimum volumes you should pipette with a 1/.1 ml pipette?

3. What (at least 3) precautions should you take when opening a box of sterile pipettes?

4. Know two common methods of growing microorganism. Explain their differences and similarities.

5. What precautions do you need to keep in mind when working with sterile tubes (nutrient broth or water blanks) with Morton closures?

6. Why do we perform serial dilutions in small steps and do not just transfer the amount of broth to a larger volume in one step?

7. Why are serial dilutions performed anyway?

8. Be able to visually distinguish between molds and bacteria.

9. Know how to spread microorganisms on nutrient agar plates.

10. Know common sources of contamination and how to avoid contamination

11. What question do we try to answer with the soap/cleanser investigation?

12. Name several hypotheses that could answer this question.

13. What results did you predict before the investigation? Why?

14. Compare your results with the class results. Explain discrepancies.

15. Compare your predictions with the class results. Explain discrepancies.

16. What is the purpose of the streak plating method>

17. Draw the result of a good streak plate as discussed above.